Semaphorin 7a Promotes Spreading and Dendricity in Human Melanocytes through β1-Integrins

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Described as secreted and membrane-bound proteins important for neural pathfinding, the class of proteins called Semaphorins are expressed in multiple tissue types and are involved in diverse biologic processes. In this study, we describe the function of Semaphorin 7a, a membrane-bound Semaphorin known to stimulate neurite outgrowth, on human melanocytes. We show that Semaphorin 7a is expressed by human keratinocytes and fibroblasts *in vitro* and *in vivo* and that melanocytes express Plexin C1, a receptor for Semaphorin 7a. Upregulation of Semaphorin 7a was observed in fibroblasts treated with UV irradiation, a potent stimulus for melanocyte dendricity. Because of the importance of melanocyte dendrites in cutaneous photoprotection, we performed functional studies examining the effect of Semaphorin 7a in melanocyte dendrite formation. We also examined the contribution of β1-integrin and Plexin C1 receptor signaling in mediating effects of Semaphorin 7a in melanocytes. We show that Semaphorin 7a induces significant melanocyte spreading and dendricity in human melanocytes. Furthermore, we show that β1-integrins and Plexin C1 receptors are ligands for Semaphorin 7a, and that signaling by these receptors has opposing effects on Semaphorin 7a-induced dendrite formation.

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INTRODUCTION

Semaphorins are a large class of secreted and membrane-anchored proteins that play a critical role in neuronal pathfinding and axon guidance in selected areas of the developing nervous system (Tamagnone and Comoglio, 2004; Yazdani and Terman, 2006). Although their expression and function were originally described in the brain and spinal cord, Semaphorins are now known to be expressed widely and have diverse functions including immune cell modulation, bone remodeling, and drug resistance (Yamada *et al.*, 1997; Harper *et al.*, 2001; Kessler *et al.*, 2004; Tamagnone and Comoglio, 2004; Delorme *et al.*, 2005; Li *et al.*, 2006; Yazdani and Terman, 2006). Semaphorins are subdivided into eight subfamilies: two are found in invertebrates, one in viruses, and five in vertebrates. The prototypical Semaphorin, Semaphorin 3A/collapsin-1, causes growth cone collapse of sensory neurons through remodeling of cytoskeletal proteins, and the majority of Semaphorins described to date act as repellant signals to neurons (Eickholt *et al.*, 1997; Gallo and Letourneau, 2004). Semaphorins bind to receptors of the Plexin family and Neuropilins serve as co-receptors (Fujisawa, 2004). Although the mechanisms underlying cytoskeletal remodeling in response to receptor activation remain to be determined, Plexin A1, a Semaphorin 3A receptor, binds directly to the guanosine triphosphate-binding protein Rac to mediate growth cone collapse (Turner *et al.*, 2004).

Semaphorin 7a (Sema7a) was first identified in 1998 and has high-sequence homology with alcelaphine herpersvirus-1 (Sato and Takahashi, 1998; Xu *et al.*, 1998). Sema7a is the first glycosylphosphatidylinositol-linked Semaphorin identified to date (Sato and Takahashi, 1998; Angelisova *et al.*, 1999; Yamada *et al.*, 1999). Unlike secreted Semaphorins, Sema7a does not bind to Neuropilin 1 or Neuropilin 2, but has been shown to bind to Plexin C1 (Tamagnone *et al.*, 1999) and the integrin β1-receptor (Pasterkamp *et al.*, 2003). Unlike most Semaphorins, which inhibit neurite outgrowth, Sema7A enhances central and peripheral axon growth and is required for proper axon tract formation during embryonic development (Pasterkamp *et al.*, 2003). A novel role for integrin receptors in Sema7a signaling was demonstrated by inhibition studies with β1-integrin inhibitory antibodies, which blocked Sema7a-dependent neurite outgrowth. In these studies, Sema7a binding to the β1-integrin receptor also activated microtubule-associated protein kinase (Pasterkamp *et al.*, 2003). Integrins are a large family of heterodimeric proteins consisting of β- and α-chains that are universally expressed and mediate cell attachment, spreading and cell migration on extracellular matrix proteins (Danen, 2005).

The expression and function of Semaphorins in the skin is only beginning to be examined. Semaphorin 3A is expressed by keratinocytes and regulates keratinocyte migration through the Neuropilin 1 receptor in an autocrine fashion, and regulates vascular morphogenesis (Serini *et al.*, 2003; Bussolino *et al.*, 2006; Kurschat *et al.*, 2006). Melanocytes

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are neural crest-derived cells and are located at the basal epidermal layer in close association with keratinocytes, which play an important role in regulating their behavior. Dendrites are a phenotypic hallmark of melanocytes, and similar to neuronal cells, melanocyte dendrites exhibit extreme plasticity, with marked variation in length and number in response to different growth factors (Imokawa, 2004). Analogous to the way in which neural cells seek out target neurons, melanocyte dendrites form growth cone-like structures that attach to keratinocytes (Scott *et al.*, 2002). The most important stimulus for melanocyte dendricity is UV irradiation, which stimulates the release of paracrine factors from keratinocytes and fibroblasts that increase melanocyte dendrite length and number, as well as melanin content (Imokawa *et al.*, 1995; Shishido *et al.*, 2001; Imokawa, 2004). UV irradiation-inducible factors that stimulate melanocyte dendricity include endothelin-1, α-melanocyte-stimulating factor, secretory phospholipase-IIA type-X, and the prostaglandins PGE_2 and $PGF_2\alpha$ (Imokawa *et al.*, 1995; Shishido *et al.*, 2001; Scott *et al.*, 2005, 2006*a*, *b*).

Melanocytes express the α3-, α6-, αv-, and β1-integrin in skin *in vivo*, and in culture the α3β1-, α5β1-, α6β1-, and αvβ3-heterodimers have been detected (Scott *et al.*, 1992; Zambruno *et al.*, 1993; Hara *et al.*, 1994). As in other cell types, integrins function in adhesion, spreading, and migration of melanocytes on extracellular matrix ligands (Scott *et al.*, 1992; Zambruno *et al.*, 1993; Hara *et al.*, 1994). The β1-integrin is expressed in a diffuse pattern along the melanocyte dendrite (Hara *et al.*, 1994), and the β1- and β3-integrins have been shown to stabilize dendrites in the epidermis, although their ligand is unknown (Hara *et al.*, 1994). Because of the identification of integrins as receptors important for melanocyte dendricity, and because Sema7a stimulates axon outgrowth through the β1-integrin receptor, we were interested to determine if Sema7a could be a factor for melanocyte dendrite formation in the skin. In this study, we explored the expression, regulation, and function of Sema7a and the Plexin *C1* receptor in the skin and the potential role of Sema7a in melanocyte dendricity. Our results show that Sema7a is a paracrine and UV irradiation-inducible ligand for Plexin *C1* and the melanocyte β1-integrin receptor. Furthermore, we show that the action of Sema7a on melanocytes dendrite formation is through the opposing signaling of the Plexin *C1* and β1-integrin receptors.

RESULTS

Sema-7a and Plexin C1 are expressed in the skin

Total RNA was extracted from cultured human keratinocytes, melanocytes, and fibroblasts. Reverse transcription-PCR was performed for detection of Sema7a and Plexin C1 message (Figure 1a). PCR products of the expected size were detected in all cell types for Sema 7a and Plexin C1. Verification of the bands with restriction digest confirmed their identity (data not shown). Comparative analysis of Sema7a levels between cell types by real-time PCR showed that the relative Sema7a expression was keratinocytes >fibroblasts >melanocytes. Sema7a protein was detected on the surface of keratinocytes (average of 10–20%) with about 60% cell surface staining of fibroblasts by flow cytometry (Figure 1b). Melanocytes did not express detectable Sema7a by flow cytometry analysis.

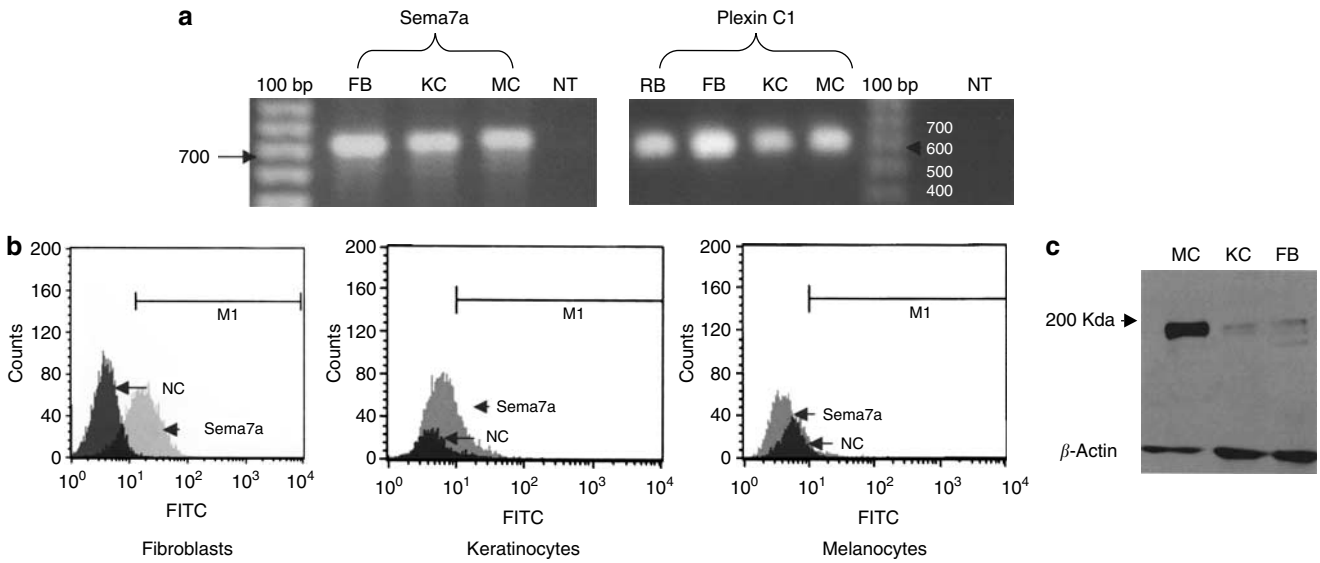

Figure 1. Expression of Sema7a and Plexin C1 in cultured fibroblasts, keratinocytes, and melanocytes. (**a**) Bands of the expected size and confirmed by restriction enzyme digest were obtained by reverse transcription-PCR for Sema7a and Plexin C1 from cultured human fibroblasts (FB), keratinocytes (KC), and melanocytes (MC). For Plexin C1 reverse transcription-PCR, rat brain (RB) served as a positive control. A 1% agarose gel stained with ethidium bromide is shown; NT indicates no template. Analysis was performed on at least two separate individual cultures that gave the same result. (**b**) Surface expression of Sema7a was examined by FACS analysis on cultured human fibroblasts, keratinocytes, and melanocytes. Representative results from six separate analyses on individual cultures are shown. (**c**) Expression of Plexin C1 was analyzed on whole-cell lysates by SDS-PAGE. A 7.5% gel in which 10 μg of total cell lysate from melanocytes (MC), fibroblasts (FB), and keratinocytes (KC) was resolved and blotted with antibodies to Plexin C1 is shown. Melanocytes express abundant Plexin C1 and a faint band is detectable in cultured fibroblasts and keratinocytes.

Comparative analysis of Plexin C1 message levels between cell types by real-time PCR showed that the relative level of Plexin C1 expression was melanocytes ≫ keratinocytes = fibroblasts. Plexin C1 protein expression, analyzed by Western blotting of total cell lysates, showed a band with the appropriate size in melanocytes, with faint but detectable bands in keratinocyte and fibroblast cell lysates (Figure 1c).

Immunofluorescence microscopy of the skin showed that Sema7a is expressed in a punctate pattern on the cell membrane of basal and supra-basal keratinocytes, consistent with its glycosylphosphatidylinositol-linked property (Figure 2a). Double labeling of sections with antibodies against Sema7a and Mel-5 showed no expression of Sema7a in melanocytes. Endothelial cells lining blood vessels in the dermis strongly expressed Sema7a, and scattered staining within spindled cells within the dermis, consistent with fibroblasts, was also evident. Our preliminary experiments showed that the Plexin C1 antibody does not work using immunofluorescence on either frozen sections or paraffin-embedded sections of skin. Therefore, we stained directly contiguous sections of skin with either Plexin C1 antibodies or antibodies to Mel-5 using avidin–biotin–peroxidase immunohistochemistry (Figure 2b). Plexin C1 staining was present in dendritic cells above the basement membrane, consistent with Langerhan's cells. Plexin C1-positive cells with peri-nuclear halos that were Mel-5-positive in contiguous sections were present, indicating expression of Plexin C1 in some, but not all, melanocytes. To examine Plexin C1 localization in melanocytes in cells *in vitro*, immunofluorescence microscopy on cultured melanocytes was performed (Figure 2c). Plexin C1 was present along the entire length of the melanocyte dendrite, but was concentrated at the tips.

Sema7a is regulated by UV irradiation in fibroblasts

UV irradiation affects melanocytes primarily through up-regulation of growth factors and receptors that stimulate

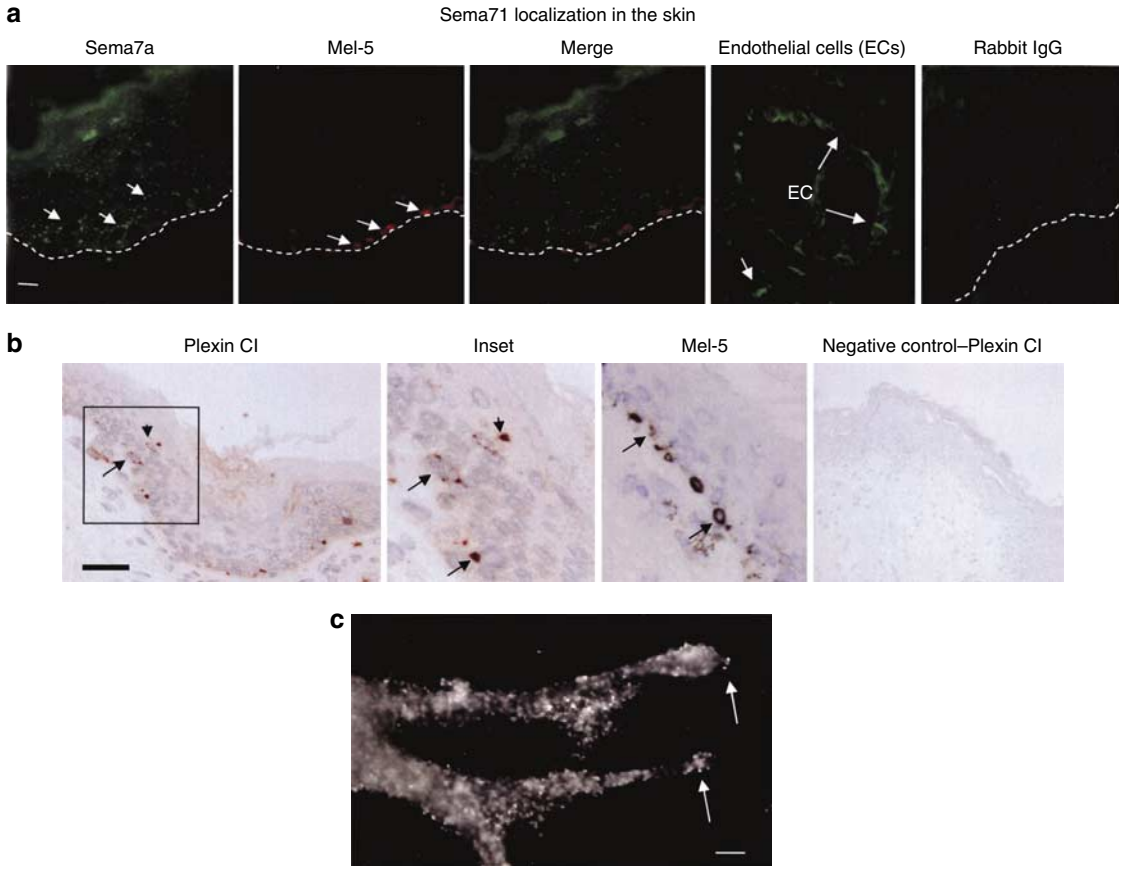

Figure 2. Expression of Sema7a and Plexin C1 in human skin *in vivo*. (**a**) Immunofluorescence microscopy of human skin double labeled with antibodies to Sema7 and Mel-5 show expression of Sema7a in the basal and suprabasal epidermal keratinocytes on the cell membrane that is somewhat punctate (arrow heads). Double labeling with antibodies against Mel-5 to identify melanocytes show individual melanocytes along the basement membrane zone (arrows). The merged images, however, do not show colocalization of Sema7a in melanocytes (Merge). Endothelial cells (EC) lining blood vessels are strongly positive for Sema7a, as well as dendritic cells in the dermis, consistent with fibroblasts (arrowheads). Sections stained with purified rabbit IgG instead of Sema7a show little or no nonspecific staining. Hatched white lines indicate the basement membrane zone. Bar = 10 μm. (**b**) Immunohistochemical staining of frozen skin sections with antibodies to Plexin C1 shows staining of solitary cells within the basal epidermal layer (arrows and inset), as well as cells above the basal epidermal layer consistent with Langerhans cells (arrowhead). Sections immediately adjacent were stained with antibodies against Mel-5 to determine if Plexin C1 is expressed in melanocytes. Some, but not all of the melanocytes, express Plexin C1. Negative controls for Plexin C1 consisted of sections stained with purified goat IgG instead of Plexin C1. Bar = 50 μm. (**c**) Cultured human melanocytes stained for Plexin C1 by immunofluorescence microscopy show a punctate dot-like pattern of staining along the length of the dendrite with a concentration at the tip (arrows). Bar = 5 μm.

dendricity and pigmentation (Imokawa *et al.*, 1995; Chakraborty *et al.*, 1999; Imokawa, 2004); hence, we next examined the effect of UV irradiation on Sema7a and Plexin C1 levels. Cells were irradiated with a single dose of light (UVA, 2.0 J/cm^2; UVB, 10 mJ/cm^2) and the expression of Sema7a and Plexin C1 was analyzed 6, 18, and 24 hours later. We chose this dose and spectrum of UV irradiation because it induces dendricity in melanocytes (Scott and Cassidy, 1998; Scott *et al.*, 2006*b*) and because this light source closely simulates natural sunlight (Figure S1). Irradiated fibroblasts showed an increase in Sema7a message at 6 hours after UV irradiation (1.8-fold), which peaked at 18 hours (fourfold) following irradiation (Figure 3a). Cell surface expression of Sema7a increased sixfold in 18 hours following UV irradiation, and remained elevated (fivefold) 24 hours following UV irradiation (Figure 3a). Keratinocytes did not show any statistically

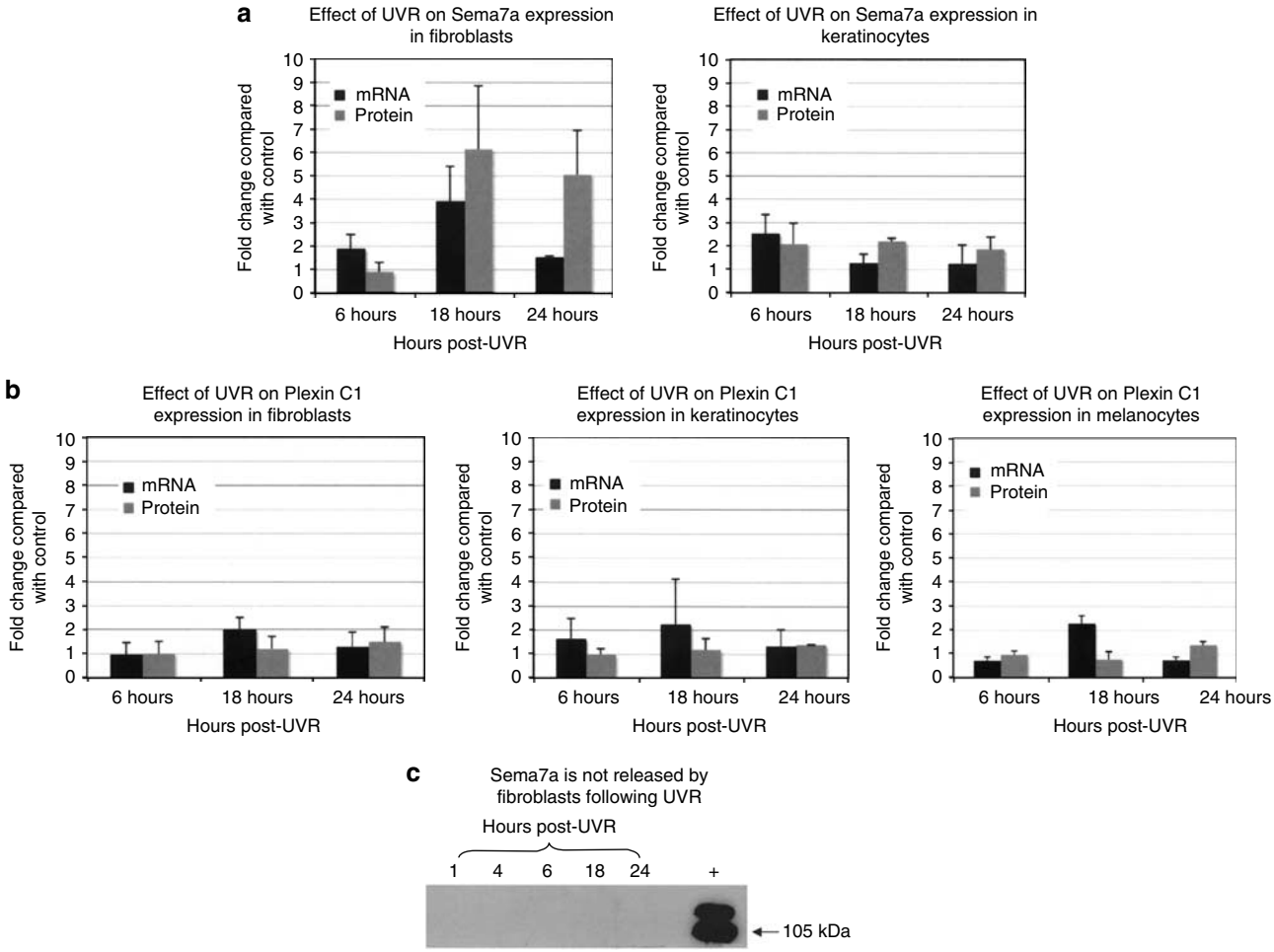

Figure 3. Regulation of Sema7a and Plexin C1 by UVR. (**a**) Sema7a is regulated by UV irradiation in fibroblasts: real-time PCR and flow cytometry were performed for analysis of Sema7a message and protein expression in UVR-treated fibroblasts and keratinocytes. Following a single dose of UV irradiation, RNA was harvested at 6, 18, and 24 hours later. Quantitative comparative real-time PCR using β-actin as a house-keeping gene was then performed. Duplicate dishes were harvested by trypsinization and stained for Sema7a and analyzed by FACS analysis. The averaged fold change (*y* axis) in Sema7a message levels and protein expression compared with Sham-irradiated controls from three separate cultures are shown. Fibroblasts show an increase in Sema7a message level 6 hours after UV irradiation (black bar) and remains elevated 24 hours after UV irradiation. Surface expression of Sema7a increased over sixfold 18 hours after UV irradiation (gray bar) and remained elevated (fivefold) 24 hours later. In contrast, irradiated keratinocytes did not show a significant change in Sema7a levels. Results are the average of three separate experiments for fibroblasts and four separate experiments for keratinocytes $\pm$SD. Fibroblasts and keratinocytes were used at passage 2–3. (**b**) Plexin C1 is not regulated by UV irradiation: real-time PCR and Western blotting were performed for analysis of Plexin C1 in UV irradiation-treated fibroblasts, melanocytes, and keratinocytes. Following a single dose of UV irradiation, RNA was harvested 6, 18, and 24 hours later and quantitative comparative real-time PCR, using β-actin as a house-keeping gene, was performed. Duplicate dishes were harvested by trypsinization and resolved in 7.5% SDS-PAGE and blotted for Plexin C1 and β1-actin. Densitometry of digital images of blots normalized for β1-actin were performed. The averaged fold change (*y* axis) in Plexin C1 message levels and protein expression compared with Sham-irradiated cells is shown. There were no significant changes in message or protein expression for Plexin C1 in the three cell types. Results are the average of three separate experiments for fibroblasts and keratinocytes and two separate experiments for melanocytes $\pm$SD. Fibroblasts, keratinocytes, and melanocytes were used at passage 2–3. (**c**) Irradiated fibroblasts do not release Sema7a into the culture supernatant: fibroblasts were irradiated with a single dose of UV irradiation and supernatant was collected 1, 4, 6, 18, and 24 hours later, and 20 μg was resolved on 7.5% SDS-PAGE and blotted with antibodies against Sema7a. Keratinocyte cell lysate (20 μg) served as a positive control. A band of 105 kDa, corresponding to the molecular weight of Sema7a, was detected in the positive control. The higher band may represent glycosylated protein. No detectable Sema7a was present in supernatants from irradiated fibroblasts.

significant increase in Sema7a at the message or protein level following UV irradiation (Figure 3a). Melanocytes, which did not express detectable Sema7a at the protein level, did not show an induction of Sema7a following UV irradiation (data not shown). Neither message nor protein levels of Plexin C1 showed statistically significant regulation in melanocytes, fibroblasts, or keratinocytes (Figure 3b). To determine if UV irradiation results in release of Sema7a into culture supernatant of fibroblasts, cells were irradiated as described above, and culture supernatant was collected, concentrated, and blotted for Sema7a (Figure 3c). The specificity of the antibody used for Western blotting was verified using blocking peptides (Figure S2). No detectable Sema7a was present in culture supernatants, indicating that Sema7a is not released from fibroblasts following the dose of UV irradiation used in these experiments.

Sema7a stimulates melanocyte attachment, spreading, and dendrite outgrowth in human melanocytes

We first employed a co-culture assay of melanocytes and 293-Sema7a cells to assess the effects of Sema7a on melanocyte adhesion/spreading and dendricity. HEK-293-Sema7a cells are stable transfectants of 293 cells that express Sema7a tagged with human placental alkaline phosphatase at the N terminus of the protein. We first stained 293-Sema7a cells with antibodies to human placental alkaline phosphatase and confirmed expression of the recombinant protein in the cytoplasm and the cell surface (Figure 4a). Melanocytes were allowed to attach to a lawn of 293-Sema7a cells for 1 hour, followed by a gentle wash. Even after this brief incubation, virtually all melanocytes cultured on 293-Sema7a cells had spread and exhibited a highly complex branching network of short dendritic projections (Figure 4b). In contrast, while a similar number of melanocytes attached to 293 cells transfected with empty vector (pAP-tag4), few if any of the attached melanocytes had spread.

Because the establishment of a mature network of melanocyte dendrites in response to growth factors requires 2–3 days (personal observation and see Scott *et al.*, 2005, 2006*b*), a longer term co-culture assay was used to determine whether Sema7a regulates melanocyte dendrite formation. Culture of melanocytes on a lawn of 293-Sema7a cells for 3

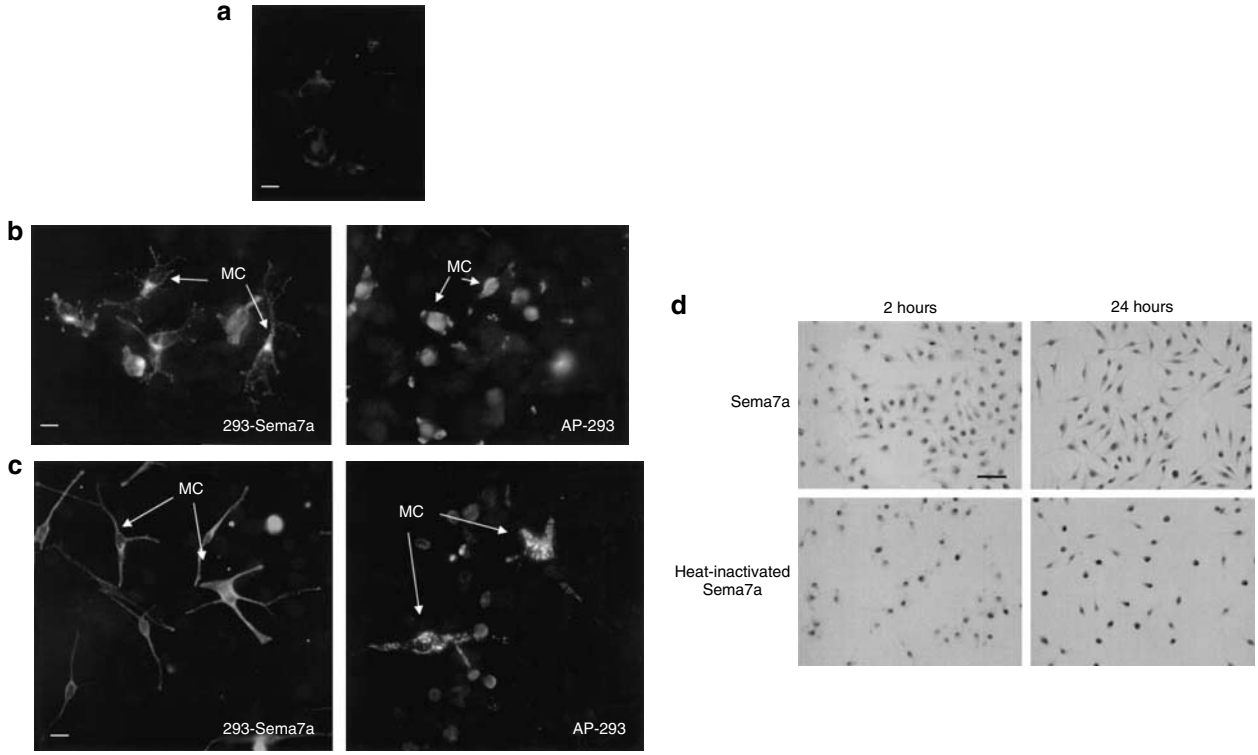

Figure 4. Sema7a stimulates spreading and dendricity in melanocytes. (**a**) 293-Sema7a cells stained with antibodies against human placenta alkaline phosphatase show expression of the recombinant Sema7a protein in the cytoplasm and focally on the cell membrane. Bar = 10 μm. (**b**) Melanocytes (MC) seeded on 293-Sema7a expressing cells or to 293 cells expressing empty vector (AP-Sema7a) for 1 hour were gently washed and adherent cells were stained with antibodies to Mel-5 to identify melanocytes and counterstained with 4′,6-diamidino-2-phenylindole to stain nuclei. Melanocytes on Sema7a expressing 293 cells attached and spread and show numerous complex branching processes. In contrast, melanocytes on 293 cells expressing empty vector (pAP-tag4) are round without evidence of spreading. Results are representative of three separate experiments. Bar = 10 μm. (**c**) MC were seeded on 293-Sema7a expressing cells or to 293 cells expressing empty vector (AP-Sema7a) for 3 days and were then gently washed, and adherent cells were stained with antibodies to Mel-5 to identify melanocytes and counterstained with 4′,6-diamidino-2-phenylindole to stain nuclei. Melanocytes grown on 293-Sema7a cells for 3 days show multiple dendrites compared with melanocytes grown on 293 cells expressing empty vector. Results are representative of six separate experiments. Bar = 10 μm. (**d**) Melanocytes were seeded on coverslips coated with recombinant Sema7a or heat-inactivated Sema7a. By 2 hours melanocytes attached and spread on Sema7a, whereas cells on heat-inactivated Sema7a were rounded. After 24 hours, melanocytes exhibited long dendrites on Sema7a, whereas melanocytes on heat-inactivated Sema7a had short blunt dendrites, or lacked dendrites entirely. Bar = 150 μm.

days resulted in an increase in the number of dendrites compared with melanocytes grown on 293 cells transfected with empty vector (Figure 4c). Forty-two percent (42% ±7.0 SD, $n=4$ individual cultures) of melanocytes grown on 293-Sema7a cells exhibited more than two dendrites compared with melanocytes co-cultured on control cells, in which only 10% (±2.5 SD, $n=2$ from three pooled cultures) of the melanocytes had more than two dendrites. The difference was statistically significant with a *P*-value of <0.05. We next analyzed melanocyte attachment and spreading to recombinant Fc-tagged Sema7a (Figure 4d). After 2 hours melanocytes spread on recombinant Sema7a, but not to heat-inactivated Sema7a. After 24 hours on Sema7a, melanocytes exhibited long dendrites, whereas melanocytes on heat-inactivated Sema7a remain rounded and had shorter dendrites.

Attachment and spreading of melanocytes on Sema7a is mediated by integrins

We next determined if β1-integrin receptors mediate the effects of Sema7a on melanocyte spreading. We confirmed that melanocytes express the β1-integrin *in vivo*, as reported by others (Halaban, 1991; Zambruno *et al.*, 1993) by double labeling of melanocytes in human skin with antibodies against β1-integrin and the c-kit receptor. Multiple areas of β1-integrin expression were detected along melanocyte dendrites and the melanocyte cell body (Figure 5a). We next tested the effects of Echistatin on Sema7a-dependent adhesion and spreading of melanocytes. Echistatin is a 49-amino-acid protein with an Arg-Lys-Asp (RGD) sequence and is a member of the disintegrin family that occurs in venoms of *Echis carinatus*. Echistatin specifically inhibits β1- and β3-integrins (Pfaff *et al.*, 1994). Pretreatment of melanocytes with Echistatin (0.1 μM) for 5 minutes before co-culture with 293-Sema7a cells completely blocked spreading of the melanocytes to the 293-Sema7a expressing cells (Figure 5b). In contrast, virtually all melanocytes treated with diluent instead of Echistatin had spread on 293-Sema7a cells (Figure 5c). We next tested the effect of functional blocking antibodies to the β1-integrin receptor (CD29) on melanocytes attachment and spreading to 293-Sema7a cells. Melanocytes were pretreated with blocking β1-integrin antibodies (0.1, 0.5, or 10 μg/ml) for 10 minutes and allowed to attach to 293-Sema7a cells for 1 hour. Controls comprised melanocytes incubated with mouse IgG. Blocking β1-integrin antibodies at all doses completely inhibited spreading of melanocytes on 293-Sema7a cells, although cells attached. Figure 5d shows representative photographs of melanocytes pretreated with blocking antibodies to the β1-integrin (0.1 μg/ml) that have attached but show no spreading on 293-Sema7a cells. In contrast, all melanocytes incubated with mouse IgG (Figure 5e) attached and spread on 293-Sema7a cells. Results for 0.1 and 0.5 μg/ml doses of blocking β1-integrin antibodies were similar and are not shown.

Plexin C1 receptor signaling inhibits melanocyte attachment and spreading on Sema7a

To determine the contribution of the Plexin C1 receptor on Sema7a-dependent dendrite formation, Plexin C1 was silenced and dendricity of control (scrambled siRNA) and Plexin C1-silenced melanocytes on coverslips coated with recombinant Sema7a or heat-inactivated Sema7a was determined. Plexin C1 silencing was verified through Western blotting for Plexin C1 (Figure 6a). Melanocytes transfected with scrambled siRNA and siRNA to Plexin C1 both showed

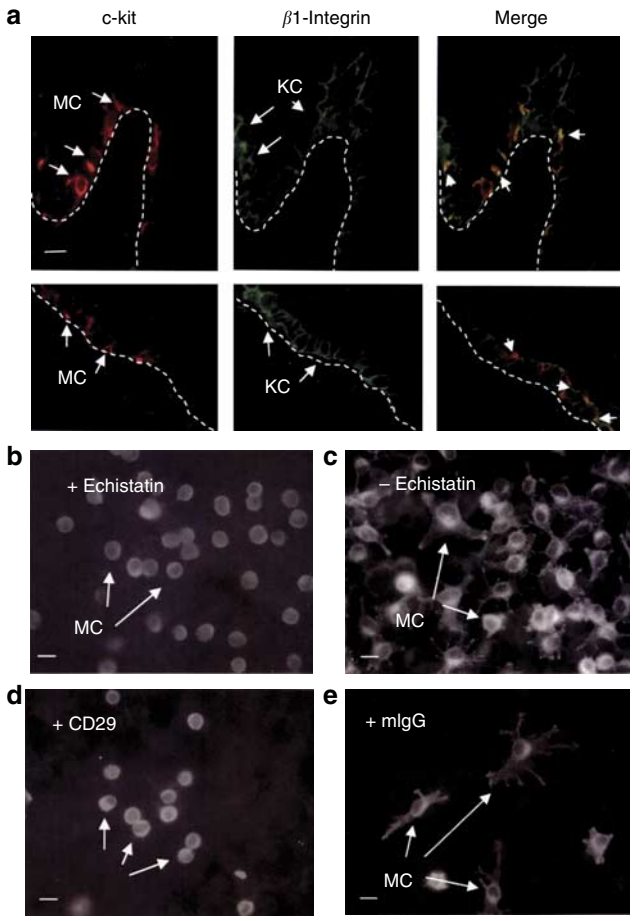

Figure 5. Integrins are receptors for Sema7a in human melanocytes. (**a**) Human skin double labeled for β1-integrin and melanocytes (MC; c-kit) show localization of β1-integrin receptors along the melanocyte dendrites, in close contact with keratinocytes (KC; arrowheads in merged image). β1-integrins are also expressed in a "chicken wire" pattern uniformly on basal keratinocytes, in a distribution similar to Sema7a (Figure 2a). Dotted line indicates the basement membrane zone. Bar = 10 μm. (**b** and **c**) MC were pretreated with Echistatin (0.1 μM) for 10 minutes before addition to monolayer culture of 293-Sema7a cells (**b**). Controls consisted of melanocytes treated with diluent instead of Echistatin (**c**). Slides were stained with Mel-5 to identify melanocytes and 4′,6-diamidino-2-phenylindole to stain nuclei. Cells treated with diluent showed the expected attachment and spreading on 293-Sema7a cells; cells pretreated with Echistatin had attached but did not show any spreading. Results are representative of three separate experiments. Bar = 10 μm. (**d** and **e**) MC were incubated with blocking antibodies (CD29) to the β1-integrin receptor (**d**: 0.1 μg/ml) for 10 minutes before addition to monolayer cultures of 293-Sema7a cells. Controls consisted of melanocytes treated with mouse IgG (**e**). Slides were stained with Mel-5 to identify melanocytes and 4′,6-diamidino-2-phenylindole to stain nuclei. Melanocytes incubated with β1-integrin blocking antibody attached but did not spread on 293-Sema7a cells. Melanocytes incubated with mouse IgG attached and spread on 293-Sema7a cells. Results shown are representative of three separate experiments. Bar = 10 μm.

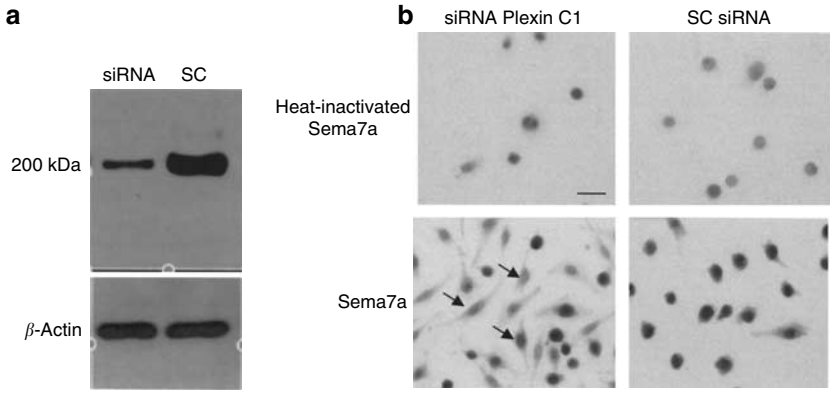

Figure 6. Plexin C1 is an inhibitory receptor for melanocyte spreading on Sema7a. (**a**) Western blot for Plexin C1 in melanocyte cell lysates transfected with either siRNA to Plexin C1 or a scrambled siRNA (SC). A 7.5% SDS-PAGE gel is shown. β-Actin served as a loading control. After 48 hours, levels of Plexin C1 protein were decreased by over 50%. Results are representative of five separate experiments. (**b**) Melanocytes in which Plexin C1 was silenced, or melanocytes transfected with scrambled siRNA, were seeded on coverslips coated with Sema7a or heat-inactivated Sema7a, for 2 hours. As expected, melanocytes adhered to and spread on Sema7a, compared with heat-inactivated Sema7a. Strikingly, melanocytes with lower Plexin C1 receptor levels showed greater spreading on Sema7a (arrows) compared with melanocytes with native levels of Plexin C1 (bottom panels). Results are representative of two separate experiments using pooled cultures of melanocytes from at least three individual cultures. Bar = 100 μm.

enhanced attachment and some spreading to Sema7a compared with heat-inactivated Sema 7a (Figure 6b). Strikingly, cells in which Plexin C1 was decreased showed a markedly increased attachment and spreading on Sema 7a compared with cells transfected with scrambled siRNAs. These data indicate that Sema7a is a ligand for Plexin C1 and that Plexin C1 activation is inhibitory for melanocyte spreading on Sema7a.

DISCUSSION

We were stimulated to investigate the role of Sema7a on human melanocytes because the pathfinding behavior of neural cells, in which dendrites make synaptic connections with target cells, is similar to the behavior between melanocytes and keratinocytes, in which dendrite extension and subsequent contact with the keratinocyte cell membrane is required for pigment donation to occur. We show that Sema7a is expressed by keratinocytes and fibroblasts *in vivo* and *in vitro,* and that its expression is upregulated by UV irradiation at the protein and message level in fibroblasts. Furthermore, we show that melanocytes express Plexin C1, a receptor for Sema7a, and that Sema7a stimulates melanocyte dendricity through the action of Plexin C1 and β1-integrin receptors that inhibit and stimulate melanocyte dendricity, respectively. Therefore, Semaphorins, which act as repellent and attractive guidance molecules in the nervous system, are candidate molecules for the regulation of melanocyte function in the skin.

We detected Sema7a message in each of the three cell types with corresponding cell surface expression confirmed in keratinocytes and fibroblasts by FACS analysis. We consistently amplified Sema7a PCR product from melanocytes, despite the absence of detectable Sema7a protein *in vivo* and *in vitro*. We cannot exclude the possibility that contaminating keratinocytes or fibroblasts were present in the melanocyte cultures used for PCR; however, the presence of Sema7a message was highly reproducible in cultures judged to be pure by inspection of cell morphology. Possible explanations are that the half-life of Sema7a in melanocytes is very short or that translational silencing of the message is occurring. In skin *in vivo,* Sema7a was identified on the cell membrane of basal and suprabasal keratinocytes. This expression pattern is similar to that of protease-activated receptor-2, a protease-activated receptor that we and others have shown is important for melanosome uptake by keratinocytes (Sharlow *et al.*, 2000; Seiberg *et al.*, 2000*a*, *b*; Scott *et al.*, 2001, 2003). Staining of skin demonstrated β1-integrin expression on the melanocyte cell body and along the length of the dendrites, in contact with keratinocyte membranes. The distribution of Sema7a on basal and suprabasal keratinocytes and the presence of β1-integrins on melanocyte dendrites suggest the possibility that Sema7a-β1-integrin may function as a ''ligand-receptor'' complex for melanosome uptake by keratinocytes, as well as for stimulation of dendrite outgrowth and stabilization. We show for the first time that Plexin C1 is expressed by melanocytes *in vivo* and *in vitro*. In fact, Plexin C1 was expressed in large amounts both at the message and protein levels in melanocytes. The localization of Plexin C1 at dendrite tips in melanocytes *in vitro* is intriguing and suggests a possible role for this receptor in melanosome transfer, dendrite pathfinding, or both.

Keratinocytes are the primary cell implicated in the regulation of melanocyte function, including growth, pigmentation, and dendricity (Halaban *et al.*, 1988*a*, *b*; Scott *et al.*, 1991; Scott and Haake, 1991; Seiberg *et al.*, 2000*b*; Seiberg, 2001; Imokawa, 2004); but paracrine factors produced by fibroblasts, such as hepatocyte growth factor and stem cell factor, influence melanocyte pigmentation (Shishido *et al.*, 2001). The biological significance of Sema7a expression by fibroblasts in melanocyte function is unclear, because fibroblasts do not directly contact melanocytes in the skin. While a soluble form of Sema7a exists *in vivo* through proteolysis of the native protein (Holmes *et al.*, 2002) we

failed to detect Sema7a in culture supernatants of irradiated fibroblasts. However, we cannot exclude the possibility that other factors, such as inflammatory mediators released by constituent cells of the dermis or chronic exposure to UV irradiation, may result in proteolysis of Sema7a, resulting in enhancement of melanocyte dendricity.

We observed a modest yet consistent upregulation of Sema7a in fibroblasts at the message and protein levels following a single dose of UVA. There are few published data on the regulation of Semaphorin expression; however, a study analyzing Sema4D regulation showed elevation of this Semaphorin following spinal cord injury (Moreau-Fauvarque *et al.*, 2003). Similarly, unpublished data from Pasterkamp *et al.* (2003) reported that Sema7a is upregulated in response to injury. Because UV irradiation exposure to the skin initiates an injury response that includes release of prostaglandins and cytokines (Snyder, 1976; Kupper *et al.*, 1987; Hunt *et al.*, 2006; Kabashima *et al.*, 2006), Sema7a upregulation in dermal fibroblasts may be secondary to one or more of these factors. Sema7a is a potent immunomodulatory protein that negatively regulates T-cell function and results in activation of monocytes (Holmes *et al.*, 2002; Czopik *et al.*, 2006). The identification of Sema7a on fibroblasts and endothelial cells in the skin *in vivo* suggests a potential role for Sema7a in the regulation of immune response(s) of the skin following UV irradiation.

Functional assays examining melanocyte morphology on recombinant Sema7a, and to 293 cells stably expressing Sema7a, showed that Sema7a is a ligand for melanocyte attachment and spreading. The rapid spreading of melanocytes on Sema7a resembled the effect of fibronectin on melanocyte spreading, which we have shown previously to be mediated by β1-integrins (Scott *et al.*, 1992). The Sema domain of Sema7a contains an Arg-Gly-Asp (RGD) binding site and three-dimensional rendition of two other Semaphorins, Sema4D and Sema3A, show that the shape of the Sema domain is organized in a similar manner to the α-integrin subunit (Love *et al.*, 2003; Pasterkamp *et al.*, 2003). β1-Integrin blocking antibodies completely inhibited melanocyte spreading on Sema7a, even at very low doses, showing that β1-integrin receptors mediate the stimulatory effects of Sema7a in melanocyte spreading. Previous studies have shown that β1-integrins stabilize melanocyte dendrites; however, ligands for β1-integrins, such as fibronectin, are not present within the epidermis (Prunieras *et al.*, 1983; Hara *et al.*, 1994). Our results suggest that the ligand for melanocyte integrins that stabilize melanocyte dendrites in the epidermis is Sema7a. The $\alpha5\beta1$-integrin is upregulated by UV irradiation in melanocytes (Neitmann *et al.*, 1999); therefore, it is possible that the effect of UV irradiation on melanocyte dendricity is amplified by upregulation of both the ligand (Sema7a) expressed by fibroblasts, and the cognate β1-integrin receptor on melanocytes. Expression of Sema7a closely parallels expression of the β1-integin receptor in basal keratinocytes (see Figure 5a and Peltonen *et al.*, 1989). Because Sema7a signaling stimulates microtubule-associated protein kinase activation in neurons (Pasterkamp *et al.*, 2003), Sema7a may be an autocrine and paracrine growth factor for keratinocytes and melanocytes, respectively.

Plexin C1 is expressed by human melanocytes as determined by Western blotting and real-time PCR, and was identified on melanocytes *in vivo* in skin sections. Plexin C1 is a receptor for the poxvirus A39R protein, a member of the Semaphorin family, in dendritic cells and neutrophils (Walzer *et al.*, 2005). Sema7a is not a ligand for Plexin C1 in olfactory bulb neurons because Plexin C1-deficient mice attach and extend dendrites in a similar manner compared with wild-type mice (Pasterkamp *et al.*, 2003). Our data show that Plexin C1 is a receptor for Sema7a in human melanocytes because regulation of Plexin C1 levels resulted in marked changes in adhesion and spreading to Sema7a. Our data show that silencing of Plexin C1 results in enhanced adhesion and spreading on Sema7a, similar to the effect of inhibitory antibodies to β1-integrins. In mouse dendritic cells, Plexin C1 inhibits integrin signaling resulting in decreased migration (Walzer *et al.*, 2005). It is reasonable to propose that Plexin C1 signaling in melanocytes inhibits β1-integrin-dependent attachment and spreading to Sema7a. Upregulation of β1-integrin expression may serve as a positive signal to limit the inhibitory effect of Plexin C1 on dendrite formation in human melanocytes. Finally, a previous study shows that epidermal keratinocytes express Semaphorin 3A (Kurschat *et al.*, 2006) and human melanocytes express the Semaphorin 3A receptor, Neuropilin 1 (unpublished observation). In contrast with Sema7a, Semaphorin 3A inhibits neurite outgrowth and integrin function (Pini, 1994; Serini *et al.*, 2003). We propose that the regulation of melanocyte dendrite formation in the skin may be controlled, in part, through the inhibitory and stimulatory actions of Semaphorin 3A and Sema7a, respectively, as well as opposing signaling of integrins and Plexin C1 receptors.

MATERIALS AND METHODS

Reagents

PureCol was purchased from Inamed Biomaterials (Freemont, CA) and the BCA Protein Assay kit was purchased from Pierce Chemical (Rockford, IL). Centircon tubes with a molecular weight cut-off of 20,000 were purchased from Vivascience (Hannover, Germany). Rabbit polyclonal antibodies to β-actin and goat polyclonal antibodies to Plexin C1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); horseradish peroxidase-conjugated goat anti-rabbit and rat anti-goat antibodies, FITC-conjugated anti-goat IgG and mouse monoclonal anti-human placental alkaline phosphatase antibodies were purchased from Sigma (St Louis, MO). Mouse monoclonal antibodies to Mel-5 and rabbit polyclonal antibodies to c-kit were purchased from Dako Corporation (Carpenteria, CA). Rabbit polyclonal antibodies to Sema7a and blocking peptides were purchased from Abcam (Cambridge, MA); FITC-conjugated mouse anti-human Sema7a (CDw108) and mouse IgM conjugated to FITC for flow cytometry were purchased from Serotec (Kidlington, Oxford, UK). Texas red-conjugated goat antibodies against mouse and FITC-conjugated goat antibodies against rabbit were purchased from Molecular Probes (Eugene, OR). Streptavidin-horse radish peroxidase, aminoethyl carbizol, and 3,3′-diaminobenzidine chromogen were obtained from Vector Laboratories (Burlingame, CA).

Full-range rainbow molecular weight markers were purchased from Amersham Life Sciences (Arlington Heights, IL). Chamber slides were obtained from Nalge Nunc International (Naperville, IL). Echistatin was purchased from Sigma. Silencing RNAs (siRNA) to human Plexin C1, Silencer Negative Control no. 1 siRNA, and lipofectamine were purchased from Ambion (Austin, TX). Sema7a, in frame with human Fc fragment, in pcDNA3 vector was a generous gift from Dr Ruslan Medzhitov (Section of Immunobiology, Yale University) and has been described previously (Czopik *et al.*, 2006). The APtag-4 vector was a generous gift from Dr Alex Kolokdin (Department of Neuroscience, The Johns Hopkins University School of Medicine).

Cells and cell culture

Neonatal foreskins were obtained according to the University of Rochester Research Subjects Review Board guidelines and were the source of cultured human melanocytes, keratinocytes, and fibroblasts. Human melanocytes and keratinocytes were cultured as described previously (Scott *et al.*, 2006*b*). All supplements except fetal bovine serum were purchased from Sigma. Fibroblasts and HEK-293 cells (ATCC, Rockville, MD) were cultured in DMEM (Sigma) with 10% fetal bovine serum (Cellgro, Herndon, VA). Stable 293-EBNA cells, expressing Sema7a tagged at the N terminus with human placental alkaline phosphatase (293-Sema7a), were a generous gift from Dr Alex Kolokdin and have been described previously (Pasterkamp *et al.*, 2003). Stable transfectants were maintained in DMEM with 10% fetal bovine serum and G418 (250 μg/ml; Sigma).

Purification of Fc-tagged Sema7a

Fc-tagged Sema7a was isolated from culture supernatant on HiTrap protein A HP columns (General Electric Healthcare, St. Giles, UK) as per manufacturer's instructions. Eluent was resolved on a 7.5% SDS-PAGE and blotted for Sema7a to verify its identity (data available in Figure S3). Coomassie-stained gels showed a single band corresponding to the molecular weight of Sema7a at a protein concentration of 1 μg/μl.

Cell attachment and dendricity analysis

293-Sema7a cells were plated as a ''lawn'' (5×10^5) on PureCol coated two-well chamber slides. HEK-293 cells transfected with pAP-tag4 vector (empty vector) were used as a negative control. Melanocytes were added to the wells in prewarmed melanocyte growth medium (MGM) for 60 minutes or 72 hours. After gentle washing with prewarmed media, slides were stained with antibodies to Mel-5 to identify melanocytes. The number of dendrites per cell and dendrite length (measured from the nucleus) were determined for approximately 100 cells from each experiment. Each experiment was performed at least four times.

Coating of coverslips with Fc-tagged Sema7a

Round glass coverslips of 12-mm diameter were acid-washed and coated with 0.02% poly-L-lysine for 3 h at room temperature. After the coverslips were washed with water, they were coated with 80 μg of recombinant Sema7a protein, air-dried overnight and blocked with 3% BSA in water. Controls consisted of coverslips coated with heat-inactivated (95°C for 5 minutes) Sema7a. Melanocytes were added to the coverslips in prewarmed MGM and cells were fixed in 3.7% formalin/PBS and stained with Mayers hematoxylin 2 and 24 hours later.

UV irradiation

Cells were irradiated with UVA 340 bulbs from Q-panel Lab Products (Cleveland, OH) as described previously (Scott *et al.*, 2005). The UV output was monitored by an IL1700 radiometer and SED400 sensor probe (Newburyport, MA). The UVA output was 2.64×10^{-3} W/cm^2 and the UVB output was 1.9×10^{-5} W/cm^2. The cells received a total UVA dose of 2 J/cm^2 and a total UVB dose of 10 mJ/cm^2. The wavelength spectrum of the UVA-340 lamps, compared with natural sunlight, is provided in Figure S1.

Western blotting and flow cytometry

Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid (DOC), 0.1% SDS, 50 mM Tris–HCl) with protease inhibitors (Boehringer Mannheim, Gmbt, Germany), and protein was quantitated using BSA as standard (Bio-Rad Laboratories, Hercules, CA). Protein was resolved on 7.5% SDS-PAGE gels and blotted using standard procedures. Visualization of the immunoreactive proteins was accomplished with an enhanced chemiluminescence reaction (Pierce Chemical). Densitometry analysis of blots was performed using NIH 6.2 software and normalized against β-actin. For analysis of Sema7a expression, cells were stained with FITC-conjugated antibodies against Sema7a. Negative controls consisted of cells incubated with FITC-conjugated IgM. Cells were analyzed using a FACS Caliber Machine (Becton Dickinson, Sparks, MD).

Immunofluorescence and immunocytochemical staining

For analysis of Sema7a expression in skin *in vivo*, human foreskins were snap-frozen in liquid nitrogen and sections were fixed in 3.7% formalin/PBS and permeabilized for 10 minutes with 0.1% Triton X-100 in PBS and blocked with 5% BSA/0.1% Triton X-100/PBS followed by incubation with antibodies to Sema7a (10 μg/ml) overnight at 4°C. For colocalization of Sema7a and melanocytes, slides were also incubated with antibodies to Mel-5 followed by the appropriate FITC or Texas red-conjugated secondary antibodies. Negative controls comprised slides incubated with purified rabbit IgG or mouse IgG (Sigma). To visualize Plexin C1 colocalization with melanocytes in skin sections, we used an avidin–biotin–peroxidase methodology, because immunofluorescence staining did not result in sufficient amplification of the signal for detection. Frozen sections of foreskins were cut and fixed in cold acetone for 6 minutes. Sections were treated with non-protein blocking solution (Dako) for 15 minutes and incubated with Plexin C1 in antibody stabilizer (1/100, Dako) for 45 minutes at room temperature. Sections were incubated with rabbit anti-goat IgG biotin-conjugated antibodies (Vector Laboratories) followed by Streptavidin horseradish peroxidase. The reaction was developed with aminoethyl carbizol. Negative controls consisted of goat IgG (Sigma) instead of the primary antibody. Adjacent sections (within 5 μm) were stained with mouse monoclonal antibodies against Mel-5 to identify melanocytes using a similar protocol, except that the secondary antibody was biotin-conjugated goat anti-mouse IgG antibodies (Vector Laboratories). The reactions were developed with 3,3′-diaminobenzidine. Negative controls comprised slides incubated with mouse IgG instead of the primary antibody.

Reverse transcription PCR and quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini kit (QIAgen, Valencia, CA). Reverse transcription was performed using 0.75 μg of total RNA with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories) on the Applied Biosystems ABI prism 7700 sequence-detection system (Bio-Rad iCycler). Primers used for amplification of Sema7a were as follows. Fwd: 5′-TCATCAAAGCC ACCATCG-3′ and rvs: 5′-AGCTCACATACAGCTTCCTCC-3′. The conditions were 95°C for 3 minutes (1 cycle); 95°C for 15 seconds; 54.5°C for 30 seconds; and 72°C for 90 seconds (40 cycles). Primers used for amplification of Plexin C1 were as follows. Fwd: 5′-AA CCATTGCACTGCAACC-3′ and rvs: 5′-GATTCCATCTTCAAGAA TCACG-3′. The conditions were 95°C for 3 minutes (1 cycle); 95°C for 15 seconds; 54.5°C for 30 seconds; and 72°C for 40 seconds (40 cycles). Primers used for amplification of β-actin were as follows. Fwd: 5′-CACGCACGATTTCCCGCTCGG-3′ and rvs: 5′-CAGGCTG TGCTATCCTGTAC-3′. The conditions were 95°C for 3 minutes (1 cycle); 95°C for 15 seconds; 54.5°C for 30 seconds; and 72°C for 40 seconds (40 cycles). PCR products were resolved on a 1% agarose gel. For real-time PCR cycle numbers were normalized to β-actin to arrive at an adjusted relative cycle number for each sample.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. UV spectrum of 340 bulbs closely simulates natural sunlight.

Figure S2. Verification of specificity of Sema7a antibody used for Western blotting.

Figure S3. Verification of recombinant Fc-Sema7a.

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